Antibiotic Resistance and Microbiome in Children Aged 1-59 months in Nouna, Burkina Faso

Manual of Operations and Procedures

Version 1.2, May 19, 2017

NCT number 03187834

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1. OVERVIEW

1.1 Executive Summary

The use of antibiotics has saved millions of human lives, however consumption of antibiotics can select for antibiotic resistant organisms and may lead to changes in commensal microbiome. Here, we propose a series of studies designed to estimate the effect of antibiotic consumption on antibiotic resistance and microbiome in a region of rural Burkina Faso. In Study One, we will measure antibiotic consumption at the community level and estimate the relationship between community-level antibiotic consumption and community-level pneumococcal resistance. In Study Two, we will measure changes in the intestinal and nasopharyngeal microbiome and resistome following a short course of antibiotics.

1.2 Objectives

Objective 1. Determine whether communities with higher levels of antibiotic consumption have higher prevalence of pneumococcal resistance in children under 5 years of age.

Objective 1B. Estimate the prevalence of active trachoma in a rural region of Northwestern Burkina Faso.

Objective 2. Determine the effect of treatment with antibiotics on microbiome diversity in children following a 5-day course of antibiotics.

Objective 2A. Determine the direct effect of a 5-day course of azithromycin, amoxicillin, or co-trimoxazole on intestinal and nasopharyngeal bacterial diversity in children aged 6-59 months compared to no treatment.
Objective 2B. Determine the indirect effect of antibiotic treatment of children in a household on intestinal and nasopharyngeal bacterial diversity in an untreated child aged 6-59 months.
Objective 2C. Assess the association between intestinal bacterial diversity and anthropometry in a population-based sample of children.

1.3 Study Sites

This study will take place within the Nouna Health and Demographic Surveillance Site (HDSS). The HDSS covers an area of 1,775 km², covering 103,576 individuals living in 58 villages as well as the town of Nouna. For **Objective 1**, we will include 20 villages served by 6 health centers: Bourasso, Sikoro, Labarani, Lekuy, Nokuy Bobo, and Kodougou in the southeast of the HDSS. For **Objective 2**, we will include the villages of Dara and Kamadena in the western region of the HDSS. To avoid contamination between studies, the two study areas will not overlap.

2. BACKGROUND

Azithromycin and other antibiotics are being considered for routine use in settings with high burdens of child morbidity and mortality. In Burkina Faso, the under-5 child mortality rate is 110 per 1,000 live births, which is among the highest child mortality rates in the world.[1] The major causes of child mortality in Burkina Faso are infectious: upper respiratory tract infection, diarrhea, and malaria.[1,2] Furthermore, malnutrition is a major contributor to child mortality and morbidity, with an estimated 35% of children malnourished in rural Burkina Faso.[3] Antibiotics may therefore reduce child morbidity and mortality by treating underlying (often subclinical) infections. *We propose to conduct this study in a region of rural Burkina Faso where the burden of child mortality and morbidity is high, and child deaths are attributable to upper respiratory tract infection, diarrhea, malaria, and malnutrition.*

Mass azithromycin distribution for trachoma control may lead to substantial decrease in child mortality.[4] A recent study in rural Ethiopia noted a nearly 50% decrease in child mortality in communities that had received a single mass azithromycin distribution for trachoma control. The use of antibiotics may also contribute to improved growth and nutrition outcomes for children in regions with a high burden of malnutrition.[5] Amoxicillin is used routinely as part of outpatient treatment of acute malnutrition. Randomized controlled trials of amoxicillin as adjunctive therapy for malnutrition has found that children receiving antibiotics gain more weight over an 8-week period compared to children receiving placebo.[6,7] Cluster-randomized trials of community-based management of pneumonia with co-trimoxazole found significantly lower all-cause under-5 mortality in communities receiving the co-trimoxazole intervention.[8] Amoxicillin and co-trimoxazole are also used for prevention of lower respiratory tract infections in high-risk children.[9] A recent systematic review and meta-analysis of antibiotic use in children in low- and middle-income settings found a consistent, strong association between antibiotic use and reduced prevalence of wasting and stunting, both among children with and without clinical indication for antibiotics.[5] We recently performed anthropometric analyses on children randomized to azithromycin versus no treatment for trachoma and found a statistically significant improvement in height among children treated with antibiotics (manuscript in preparation).

Millions of doses of azithromycin are distributed in predominantly rural Sub-Saharan African settings for trachoma control as part of mass azithromycin distribution programs. In addition to drastically reducing the prevalence of trachoma globally[10-16], previous work has shown that azithromycin distributed as part of trachoma control programs has led to decreases in diarrhea[17,18], skin infections, lower respiratory infection[19], and malaria[20], in addition to all-cause mortality.[4] On the other hand, the risks of mass azithromycin distribution are largely confined to non-serious adverse events, including abdominal pain, nausea, and vomiting[21], and transient increase in community resistance, a resistance which has never been shown to be clinically meaningful and resistance levels return to normal levels when antibiotic selection pressure is removed.[22,23]

There is a potential for unintended consequences with the use of antibiotics, including reduced bacterial diversity. Antibiotics may alter the host's commensal microbiota.[22,24-29] A recent study among antibiotic-naïve children in Niger found a significant decrease in bacterial

diversity in children receiving a single dose of azithromycin compared to placebo[24], although this study did not evaluate long-term changes in microbiome following an antibiotic course. To date, there have been very few randomized assessments of changes in intestinal microbiome following a course of antibiotics, and thus the causal changes in microbiome are understudied. Furthermore, any functional differences due to altered microbiome remain as-yet unknown, as microbiome studies are in their infancy and mechanistic studies are lacking. Children with malnutrition have been shown to have decreased intestinal bacterial diversity. [30], This association is thought to be a result of poor diet and increased susceptibility to infection that leads to structural changes in the gut, poor absorption of nutrients, and subsequently altered microbiome.[31] Furthermore, environmental enteric dysfunction is thought to be widespread in rural Sub-Saharan Africa. This condition is mediated in part by chronic infection with gram negative bacteria, and causes morphologic changes in the intestines that lead to malabsorption and subsequent malnutrition and stunting.[32-34] Presumptive treatment with antibiotics may modulate the association and lead to intestinal microbiome changes that are actually beneficial for child health.

We propose two studies designed to gain a better understanding of the mechanisms behind the potential benefits and consequences of antibiotic use in settings with high child mortality and morbidity. We specifically propose to study these outcomes in children aged 6-59 months, as they are by far at the greatest risk of mortality due to infectious causes and are at the highest risk of malnutrition, and thus would benefit the most from child morbidity and mortality interventions . To date, there have been very few randomized assessments of changes in the intestinal microbiome following a course of antibiotics, and thus the causal changes in microbiome are understudied. We propose two studies to 1) understand the effect of community antibiotic consumption on community antibiotic resistance and 2) the effect of a short course of routine antibiotics on changes in intestinal and nasopharyngeal microbiome and resistome. We hypothesize that communities with higher antibiotic consumption will have higher prevalence of pneumococcal resistance in nasopharyngeal samples. Furthermore, we hypothesize that a short course of antibiotics will lead to decreased bacterial diversity shortly after completion of the antibiotic course, and higher probability of identification of bacterial resistance genes in rectal and nasopharyngeal samples.

3. STUDY DESIGN

We propose two studies to address the proposed objectives. **Study One** will be a cross-sectional, observational study designed to assess the relationship between community antibiotic consumption and pneumococcal resistance prevalence. **Study Two** will be a four-arm randomized controlled trial designed to yield estimates of the effect of a short course of antibiotics on intestinal and nasopharyngeal microbiome diversity and resistome.

3.1 Study One: Community antibiotic consumption and prevalence of pneumococcal resistance

Study One is a cross-sectional assessment of community antibiotic consumption and nasopharyngeal pneumococcal resistance. The study will occur in two phases.

During Phase I, we will extract data from routinely-collected Prescription Sheet from 6 health centers located in the Nouna HDSS. These prescription sheets are collected and stored at the health facility for all children under the age of 5. The sheets are taken to the pharmacy when the caregiver fills a prescription for the child. We will extract antibiotic prescriptions (and dispensing, as these sheets are filled when the caregiver picks up the prescription) for a period covering approximately 6 months (January through June, 2017). We will extract the following data on a standardized, electronic data collection form (see Section 8, Data Collection and Management):

- Health facility name
- Date of prescription
- Village of residence of the child
- Child's age
- Child's sex
- Antibiotic
- Dose and duration of antibiotic

To calculate antibiotic consumption, we will calculate the total doses of antibiotics and standardize using the size of the village per the most recent HDSS census.

During Phase II, we will determine pneumococcal resistance in a random sample of 15 children aged 6-59 months in each village in the catchment area for each health facility (e.g., each village for which antibiotic data were collected during Phase I). Children will be randomly selected for inclusion based on the most recent HDSS census. Nasopharyngeal swabs will be collected from each randomly-selected child, which will be processed via Kirby-Bauer disc diffusion assay at the CRSN microbiology laboratory (see Section 4 for details on specimen collection and Section 6 for details on microbiological methods). In addition, we will perform conjunctival photography and clinical evaluation of the conjunctiva on each enrolled child for rapid assessment of trachoma prevalence in each village, if a child is diagnosed with active trachoma the guardian will be given a tube of tetracycline. Caregivers of children randomly selected for inclusion in the study will additionally complete a brief antibiotic use survey.

3.1.1 Sample Size

A total of 6 health facilities and 20 villages will be included in Study One. We will collect nasopharyngeal swabs from 300 children in the 20 villages. Duplicate swabs will be collected on 2 children per village, so a total of 320 swabs will be collected.

3.1.2 Health Center and Community Eligibility

All health centers in the HDSS will be eligible for inclusion. We select health centers based on their accessibility during the rainy season, and select communities in a contiguous area of the HDSS. All villages in the catchment area of the health facility that are in the HDSS will be included.

3.1.3 Individual Eligibility

Children residing in the villages in the catchment area of included health facilities during the last census who are 6-59 months will be eligible for random inclusion in the study. We will use the census to randomly select 15 children aged 6-59 months of age from each village. If the child has moved out of the village or died since the last census, we will randomly select another child from the census.

3.2 Study Two: Antibiotic treatment and microbiome changes in children

Study Two is a randomized trial of a short course of antibiotics (azithromycin, co-trimoxazole, or amoxicillin) compared to no treatment. We will assess the effect of a short antibiotic course on intestinal and nasopharyngeal bacterial diversity and will test for antibiotic resistance in the resistome. Households with at least 2 children aged 6-59 months will be randomized in a 1:1:1:1 fashion to one of four study arms: 1) azithromycin, 2) amoxicillin, 3) co-trimoxazole, or 4) no treatment. All children minus one in the household will be treated, and we will assess the indirect effect of antibiotic treatment on the untreated child residing in the household. We will measure changes in microbiome three days following completion of the antibiotic course (primary outcome). During the final sample collection we will also complete a brief survey about the children's health particularly about diarrhea and respiratory infections. Finally, 4 weeks after the completion of the treatment we will evaluate the children's growth by doing anthropometric measurements.

3.2.1 Sample Size

We will enroll 120 households into the four study arms (30/arm).

3.2.2 Baseline Measurement

After enrollment and informed consent but prior to randomization, children will undergo anthropometry (height, weight, and mid-upper arm circumference measurements). Caregivers will complete a brief baseline survey that will include information on whether the child is currently exclusively breastfeeding, is currently experiencing diarrhea (measured by the standard WHO question, "Has the child had 3 or more loose or watery stools in the previous 24 hours"), and, for non-exclusively breastfeeding children, the child's dietary diversity.

3.2.3 Randomization

A list of potentially eligible households will be generated from the most recent census. This list will be sent to the study biostatistician (TCP), who will generate the randomization list. The randomization list will be 1:1:1:1 and will be generated without stratification or blocking.

The randomization list will contain the randomization arm and household identification number and will be transmitted to the field coordinator at CRSN.

3.2.4 Community Eligibility

We will select two communities that have at last 120 households with 2 or more children under age 6-59 months at the most recent census. Children who are allergic to any of the study medications will not be enrolled in the study. Children who are receiving antibiotics for an ongoing disease won't be included in the study

3.2.5 Household Eligibility

Households will be eligible for inclusion in the study if they have 2 or more children age 6-59 months currently residing in the household.

3.2.6 Study Medication

Study medication will be procured locally for amoxicillin and co-trimoxazole. Azithromycin and placebo will be procured in the United States as they are not readily available locally, and shipped to the study site. All medications will be prepared as an oral suspension.

3.2.6.1 Dosage Information

All children will receive 5 days of treatment. Dosages will be administered once per day for all medications with the exception of amoxicillin, which will be twice per day. Dosage will be weight-based, based on the baseline weight assessment as part of anthropometry measurements, as follows:

- Azithromycin: 10 mg/kg once daily on Day 1, then 5 mg/kg once daily Days 2-5
- Amoxicillin: 25 mg/kg/day, divided into twice daily doses for Days 1-5
- Co-trimoxazole: 240 mg daily for Days 1-5
- Placebo: Once dose daily for Days 1-5

3.2.6.2 Directly Observed Treatment

All doses of study medication will be directly observed by a field worker. Study medication will be prepared on the day of administration by staff at the CRSN and will be pre-loaded into syringes with the correct dosing based on the child's weight from the baseline assessment. Each syringe will be labeled with the child's identification and household ID number. All doses of medication will be recorded electronically on the tablet.

3.2.6.3 Adverse Events Systems

All study medications are generally well-tolerated. The most common side effects of azithromycin are diarrhea, abdominal pain, and vomiting, each of which may occur in fewer than one in twenty persons who receive azithromycin. Rarer side effects include abnormal liver function tests, allergic reactions, and nervousness. Diarrhea due to *Clostridium difficile* has been reported in rare cases.
Each day, the staff member will screen all enrolled children for any adverse event. All adverse events (non-serious and severe) will be communicated to the Medical Monitor on a daily basis.

The most common side effects of amoxicillin are diarrhea, abdominal pain, and vomiting. Rach or other hypersensitivity reactions have been reported in up to 10% of patients. Diarrhea due to *Clostridium difficile* has been reported in rare cases.

The most common side effects of co-trimoxazole (sulfamethoxazole/trimethoprim) include nausea, diarrhea, and headache. Some patients may develop a rash.

During the consent process, the common adverse reactions that may occur will be explained to parents/guardians, and they will be advised to communicate any adverse events to the local health facility immediately. If, for any reason, the participant needs further care, they will be referred to the nearest health center for examination and treatment. The health facility works closely with the Nouna HDSS, and patients who are enrolled in the study will be identified based on a unique study card that will be given to each parent/guardian for each study participant. Adverse events will be reported to the study team immediately.

3.2.7 Study Schedule

	Day 0	Day 1-5	Day 9	4 weeks after treatment completion
Enrollment	X			
Anthropometry	X			X
Swabs	X		X	
Randomization*	X			
Treatment		X		
Outcome survey			X	

*After all baseline assessments are complete

4. SPECIMEN COLLECTION PROCEDURES

4.1 Nasopharyngeal Swabs

Nasopharyngeal swabs will be collected from a random sample of 15 children per village included in Study One for assessment of antibiotic resistance and from all children in Study Two for assessment of nasopharyngeal microbiome.

The examiner will:

1. Place a pediatric flocked swab with a nylon tip through the right nostril and down the nasopharynx of each participant. Note that if the swab is not perpendicular to the frontal plane of the face, it is likely not in the inferior turbinate.
2. Once you reach the nasopharynx, rotate the swab 180° as you remove the swab from the nose.
3. Place the swab in a tube containing 1.0 mL DNA/RNA shield media by Zymo or STGG (skim milk, tryptone, glucose, and glycerin) media, cut the handle off using sterile scissors, and close the cap of the tube with the swab immersed.
4. The nasopharyngeal swab samples in STGG will initially be stored in the field at 4°C using an insulated storage bag with Fisher brand ice gel packs, and then transferred to -20°C. The nasopharyngeal swab samples in DNA/RNA shield media will be stored in ambient temperature in the field. Then transferred to a refrigerator or freezer.
5. The scissors used to cut calcium alginate swabs will be sterilized with alcohol pads or cleaned with bleach wipes between participants. When collecting specimens in DNA/RNA shield, scissors will be cleaned between participants - first with bleach wipes, and then with alcohol pads.

Do not attempt to collect the NP swab if you are not successful after **three** attempts.

Nasopharyngeal swabs will be stored in DNA/RNA shield media by Zymo or STGG media, and standard microbiologic techniques will be used to isolate *S. pneumoniae* and test for resistance to erythromycin, tetracycline, sulfa-trimethoprine, oxicillin, and clindamycin. Resistant isolates will be assessed for the most common genetic resistant determinants (*ermB* and *mefA*) using a PCR-based assay. Serotype will be assessed using a nested PCR reaction for the most common serotypes, followed by the Quellung reaction for any untyped isolates.

Materials for Swab Collection for Resistance Testing

Swabs

NP specimens will be collected using sterile, individually-wrapped pediatric flocked swabs with a plastic swab shaft (manufactured by Copan). Nasal sites will be swabbed with a sterile, Dacron polyester-tipped swab with a plastic shaft (manufactured by Fisherbrand).

Sample Tubes

All field samples for DNA testing will be collected into sterile 2.0ml microcentrifuge tubes, manufactured by Sarstedt®. (DNA-free tubes will be used for collection in DNA/RNA shield.)

Cooler Bags with Frozen Ice Packs

Insulated cooler bags will be used to carry samples to and from the field. In addition, frozen gel ice packs designed to thaw slowly will be used to maintain the temperature in the cooler bags during transport.

-80°C Freezer

A standard -80°C freezer located at the CRSN microbiology laboratory will be used for the storage and freezing of ice packs and samples. This freezer is kept in a locked room on the grounds of the CRSN, which is under 24-hour security guard supervision.

Protocol for Tubing and Handling of Samples

The tubing and handling protocol must be carefully followed in order to prevent contamination and ensure the safe transport of the samples back to the CRSN microbiology laboratory and/or to the US for processing. The person in charge of labeling, tubing, arranging, and handling the samples needs to perform this task in the most orderly and attentive manner.

1. Both hands of the tuber should be gloved at all times. The tuber's gloves only need to be changed when any potential contamination of the gloves occurs. The tuber opens the capped, hinged lid of a microcentrifuge tube, which has been labeled with the participant's random identification number.
2. The swab is inserted by the examiner into the microcentrifuge tube held by the tuber. The swab shaft should only be inserted until the swab head is fully in the tube. The tuber will cut the swab shaft with sterile scissors.
3. The tuber should screw the cap of the microcentrifuge tube tightly, flick the tube to mix the sample with the media (for tubes with DNA/RNA shield media), and place it in the sample collection box, located in the cooler bag filled with frozen ice packs. The flap of the cooler bag should be closed between each patient. The cooler bag should be in as cool a place as possible in the field, in a shaded area out of the sun.

Upon returning from the field each day, the samples in STGG will be immediately taken to a local health center and stored in a commercial -80°C freezer, reserved solely for storage of specimens and ice packs. All samples will be in sample boxes, labeled with the village name for easy future identification.

4.2 Rectal Swabs

Rectal swabs will be collected and placed into Amies transport media or Norgen Stool Preservative. The test will require that the child's parent and examiners work together to obtain a good sample. Is it important to describe the test to the parent so that they can best assist with keeping the child still during the procedure, if necessary.

In place of stool specimens, rectal swabs can be collected in the following way:

1. Put on a clean pair of gloves.

2. Partially open the fecal swab package and remove the top section of the collection vial (this can be discarded).
3. Position the child:
    - Lie the child on his/her back, hold legs in the air (it is useful to have assistance).
    - Or have the child lay on his/her stomach across the mother/guardian's lap
4. Remove the swab from the package. Take care that the cotton tip is not touched. If it is touched, throw the swab away and begin with a new one.
5. Insert the tip of the swab into the child's anus only as far as needed to contact fecal material (1-3cm) and rotate 180 degrees. The tip should be a brownish color when removed.
6. Place swab into the preservative in the collection tube. Make sure the swab is fully submerged in the liquid preservative and then break the swab off using the pre-scored breaking point.
7. Screw the cap back on the tube and make sure that it's tightened. Wrap the area where the cap meets the tube with Parafilm to ensure that the sample will not leak, and then place the tube into the appropriate sample box.
    - If the swab cannot be broken off while the tip is fully submerged in the liquid, try twirling the swab in the liquid first (to release the contents of the sample into the preservative) before breaking it off. Avoid rubbing the sample on the tip of the swab off on the side of the tube where there is no liquid.
8. Place a random number label on the collection tube.
9. Place the tube the rectal swab container.
10. **Swab storage for Genetic analysis:** Store samples at room temperature. According to the manufacturer, the preservative in the tube will preserve DNA for 5 months at room temperature (7 days for RNA), and thereafter can be frozen (-20°C or -80°C) for long-term storage.

Rectal Swab Collection for Culturing/Microbiological Testing

Swab

An individually-wrapped Copan flocked swab with a plastic shaft will be used to collect the rectal swab and then placed into a Stool Nucleic Acid Collection and Transport Tube containing Norgen Stool Preservative or Amies Transport Medium.

Sample Tube with Media

The specimen will be in a sterile Stool Nucleic Acid Collection and Transport Tube containing Norgen Stool Preservative or Amies Transport Medium with a cap that will be tightened firmly.

Quality Control Measures for Specimen Collection

Negative Field Controls

Negative field control swabs for NP and stool will be taken in each community to assess for contamination: one control swab each (NP and stool/rectal) are taken before specimen collection begins in a community; and another (NP and stool/rectal) upon completion of

specimen collection. For each negative field control, the examiner will open a new swab as described above. Wave the swab in the air, without making contact with anyone/anything. Tube the swab in media, as described above.

Duplicate swabs

Duplicate NP swabs and rectal swabs/stool specimen will be collected from two children per community.

Stool samples

Each caregiver will be supplied with a bag and plastic child's potty chair and instructed to have their child defecate in a bag and return the bag to the stool collection station. Children that cannot produce a stool within two hours will be given supplies to collect the stool at home. Caregivers will be instructed to collect stool the following morning, after which a study team member would come to their house to pick up the specimen. From a single stool specimen, we will preserve two samples: 0.5 ml of stool in 0.5 ml ethanol and 1 gram of stool in 10 ml sodium acetate-acetic acid formalin (SAF) for microscopy. Stool samples preserved in ethanol and potassium dichromate samples will be placed on ice immediately after collection and transported to a -20ºC freezer by the end of the day. SAF-preserved samples will be kept at room temperature in the shade.

Supplies for stool sample collection

- Child potty
- Wooden dowls
- Digital specimen scale
- Stool specimen tube containing 10ml SAF preservative
- 2ml tubes for PCR preservation
- Applicator stick
- Alcohol wipe
- Fine permanent Marker
- Trash bag
- Gloves
- Batteries
- Sanitizing hand wipes

Laboratory methods

a. Real-time targeted PCR

DNA will be extracted from rectal swabs and stool samples using the the ZR-96 DNA Clean & Concentrator™-5 Kit (Zymo Research Corporation, Irvine, CA). DNA concentration will be measured using the Qubit 1.0 Fluorometer (Life Technologies, Carlsbad, CA) and the Qubit

dsDNA Broad Range Assay Kit (Life Technologies). Quantitative real-time polymerase chain reaction (qPCR) will be performed to for speciation and quantification of the parasite burden as previously described by Piloette et al[18]. Species-specific qPCR amplification will be conducted in 7 µl volumes, containing 3.5 µl of 2X TaqMan Fast Universal PCR Master Mix (Life Technologies), 125 nmol of each assay's respective probe, 2 µl of template DNA at a concentration of 1 ng/µl, and respective concentrations of each species-specific PCR reaction developed by Pilotte et al (see Table 2). Thermocycling conditions will be as follows: an initial 2 min incubation step at 50°C, followed by a 10 min incubation at 95°C. These incubations will be followed by 40 cycles of 95°C for 15 sec for denaturation, followed by 1 min at 59°C for annealing and extension. All reactions will be conducted using the StepOne Plus Real-Time PCR System (Life Technologies).

b. Microscopy

All sodium acetate-acetic acid formalin preserved samples will be processed at the CRSN laboratory using the diethyl ether-concentration method and examined microscopy for soil-transmitted helminth eggs and larva[24]. Briefly, the preserved specimen will be gently shaken, then filtered and centrifuged for one minute at 2000 revolutions per minute (rpm). The supernatant will be decanted and the precipitate mixed with 7 ml of 0.85% NaCl and 3 ml of diethyl ether. The whole sample will be centrifuged again for five minutes at 2000 rpm[24], the supernatant decanted again, and the sediment examined with a microscope at 10x and 40x magnification for helminth eggs and intestinal protozoa cysts. For helminths, the number of eggs will be counted and recorded, with a possible range of 1 up to >100 eggs. As quality control, an independent expert laboratory technician will confirm all positive slides, as well as every 10th negative specimen.

4.3 Conjunctival Photography and Clinical evaluation.

4.3.1 Examination position

The examining position to be used in the field for young children will be the classic pediatric ophthalmic examination technique. With the aid of a helper seated directly opposite to the examiner, the child will be positioned with his/her head between the examiner's knees, with the child's face looking upwards toward the examiner. The legs of the child will be straddled across the helper and the arms held gently across the child's chest. Care should be taken to keep the child's eyes above the level of the examiner's knees, in order to properly take the conjunctival swab.

4.3.1 Everting the upper eyelid

For all participants, only the right upper eyelid will be examined for this study. The only exception to this is if the right eye is difficult to examine due to eye disease or injury, in which case the left eye will be examined. In order to avoid passing contamination from the child into the eye, once the examiner dons a new pair of gloves, their gloved hands should not be used to position the child. The examiner uses their fingertips to grasp the central portion of the participant's upper lid eyelashes. The upper lid is then everted, using a finger of the examiner's other hand (or the end of a sterile swab) as a fulcrum, positioned superior to the tarsal plate.

The everted lid is held in place by the examiner's non-dominant hand holding the eyelashes against the orbital rim.

4.3.3 Conjunctival photography

Specific team members will be designated as photographers for the entire trial in order to ensure high quality photographs by an experienced photographer. The photographer will take photographs of the conjunctiva with a handheld Nikon D90 digital camera with a macro lens (1:1). Conjunctival photography causes no damage to the eye, is well tolerated by children, and is a standard clinical procedure at UCSF. Photographs will be identified by first taking a photograph of the 6-digit random identification sticker that was assigned to the child during the registration phase of the study visit. By convention, all photos taken after this random number (up to the next photographed random number) belong to the preceding random number. If a random number is mistakenly not photographed before taking a photograph of the conjunctiva, it may be photographed afterwards; in this case, a note should be included that says "preceding photos." Photographs are stored on the memory card in the camera, and then transferred to an external hard drive each night. Batteries are charged each night; each camera bag has at least 2 spare batteries.

Photography Protocol

- Equipment needed: camera, labels, photo log book, and CD burner
- Miscellaneous equipment: Extra battery, card reader, lens filter, extra media card

Camera Setup

A handheld Nikon D-series camera and lens will be used for all photographs. If possible, use natural light rather than a flash, as the flash introduces artifacts into the image.

- Model
  - Nikon D-series camera: D-40, 50, 70, 80, 90, 100 or 200
  - There are minor differences between the different models, mostly related to setup
- Lens
  - Nikon Macro Autofocus 105 mm f/2.8
- Settings
  - Manual setting- turn off autofocus
  - Disengage "limit" engage "full"
  - Extend lens to 1:1; note with older versions of this lens it is 1:1 at full extension; newer versions can go to slightly higher magnification than 1:1 so caution must be exerted
  - Image size/resolution
    - Jpg normal- set thru "setup" menu
    - Large- set thru "setup" menu ("small and medium" are probably adequate)
- White balance- automatic or flash; set thru "setup" menu
- Shutter- Aperture Priority
- f57- set thru menu; note if lens is not extended to 1:1, f57 will not be allowed

- ISO 400

Photograph Procedure

- Place patient into position that will allow maximum stability; standing, sitting or "head-clamp" position. Employing village volunteer to help is very useful.
- Take photo of patient ID
- Image is brought into focus by changing the working distance, not by turning the lens. This is because the lens is fixed in its manual setting. The working distance is approximately 20 cm from the eye with our current settings
- Take minimum of 2 photos. If there is any doubt of the quality of the photo while the patient is in position it is better to continue to take more photographs before the patient is allowed to leave. It is easy to delete photos if they are not needed.
- Check photos before allowing child to leave. If they are not acceptable, repeat procedure. Only stop if the patient or guardian requests that we stop, or if is deemed impossible, even with further attempts. Note we have obtained >95% acceptable photos in previous studies.
- If the child cannot be photographed for some reason, it does not affect the eligibility of the child. A notation is made on the ocular form of why the photograph cannot be taken, but no replacement is sought. Again, this is expected to be a rare event.

Photo Troubleshooting Guide

Note all camera settings are permanently embedded in every photo that is taken and can be viewed with the camera or with any standard commercial photo-viewing software (e.g., Adobe Photoshop; Photomechanic, Nikon View, Canon, etc.)

- Photos too DARK, too LIGHT or OUT OF FOCUS
    - Check camera settings: ISO 400, Aperture preferred, +/- on zero, Manual setting on lens, flash elevated; battery fully charged
- If all photos are affected in the same way than it is most likely the settings on the camera or lens
    - Change camera settings from default recommendations
    - Decrease F stop to lighten, increase to darken
    - Could be taken down to 32 or lower but depth of field will be lessened
    - +/- can be increased or decreased to change exposure (+ to lighten; - to darken)
    - Check that lens is on Manual NOT Autofocus
    - ISO- increase to lighten photo, decrease to darken
    - Very high ISO will produce graininess
    - Meter- try "spot" instead of "pattern
    - Have another person observe photography in real time
    - Make sure flash is not depressed by photographer or someone else while photo is taken. (This commonly happens if photographer has loupes). Is the lens well supported?
    - Make sure battery is fully charged

- If first photos in series are acceptable and then they gradually become less exposed (darker) it might be because the battery is gradually losing power during the session. Note that the flash reaction time increases as the battery power decreases.
- Out of focus
    - F stop may be too low; movement artifact; flash doesn't work; lens is dirty
- Image not centered
    - Movement of child or camera (hold child's head between knees of "helper"; stabilize camera with second hand)
- Reflection artifact
    - Move camera slightly between first and second photos to achieve different angle; gently dab conjunctiva with swab- must be done at periphery to avoid creating inflammation

Grading of clinical conjunctival photographs

Three experienced ophthalmologists will grade the photographs at the conclusion of the study. Graders will grade each photograph in the set for follicles and inflammation according to the WHO simplified grading system.

4.3.4 Clinical evaluation of the conjunctiva

On site clinical examination will be performed by medical staff member previously trained. The examiner will use the 2.5X magnifying ocular loupes (Wilson ophthalmics) to assess the tarsal conjunctiva of the everted upper right eyelid. The examiner will grade the conjunctiva according to the WHO simplified Trachoma grading scale (see table below). If necessary, a hand-held torch light will be used by the examiner for illumination of the conjunctiva. The examiner tells the Trachoma Grade to the tuber, who then records the grade onto the electronic device. Field staff will only be certified to grade for the study if they exceed sufficient agreement with an expert trachoma grader (kappa≥0.6 on a series of 25 pictures) during the training session.

Definitions of the WHO Simplified Trachoma Grading Scale

TF (Trachomatous Inflammation – Follicular): the presence of five or more follicles in the upper tarsal conjunctiva.
TI (Trachomatous Inflammation – Intense): pronounced inflammatory thickening of the upper tarsal conjunctiva that obscures more than half of the normal deep tarsal vessels.
TS (Trachomatous Scarring): the presence of scarring in the tarsal conjunctiva.
TT (Trachomatous Trichiasis): at least one eyelash rubs on the eyeball.
CO (Corneal Opacity): easily visible corneal opacity over the pupil.
Clinically active trachoma: *defined as either TF or TI. If WHO guidelines recommend that TF alone is the most appropriate sign to follow, then we will easily be able to report this.*

5. ANTHROPOMETRY

Anthropometry will be collected for all children in Study Two at baseline and 4 weeks after the completion of the treatment. We will measure length or height, weight, and MUAC.

Measuring Length and Height
A lightweight measuring board will be used to measure the participant's height to the nearest 0.1 cm. Height will be assessed with a ShorrBoard. Depending on a child's age and ability to stand, measure the child's length or height.

Length: If a child is less than 2 years old, or cannot stand alone, measure recumbent length. A child's length is measured lying down (recumbent).
Height: If a child is able to stand, we will measure standing height.

Procedure
If the child has braids or hair ornaments that will interfere with length/height measurements, remove them if possible. Check that any sandals, shoes, or socks have also been removed. Whether measuring length or height, the mother/guardian is needed to help with measurements and to soothe and comfort the child. Explain to the mother the reasons for the measurements, and describe the steps in the procedure. Answer any questions she might have. Show her and tell her how she can help you. Explain that it is important to keep the child still and calm to obtain the best measurement.

ShorrBoard Set-Up

1) Remove ShorrBoard from bag.
2) Stand the ShorrBoard upright. You can step on the base of the board to keep it stable.
3) As you face the board, turn the non-removable bolt counterclockwise to release the extension piece. Note: the bolt remains attached to the back of the extension piece – do NOT remove it.
4) Slide the extension piece into the top end of the main board and fasten the clasp on the back of the board. Make sure the clasp is fastened properly.
5) The auto-lock sliding head/footpiece is stored in the base of the main board and can be moved up and down the length of the measuring board. It should stay in place on its own wherever you position it.
6) The measuring board must be placed against a firm surface for standing height (e.g. wall, table, tree, against a vehicle, etc.). Make sure the board is stable. If necessary, place items such as small rocks underneath the height board to stabilize it during the measurement.
7) Clean the equipment with alcohol swabs at the beginning of each day.

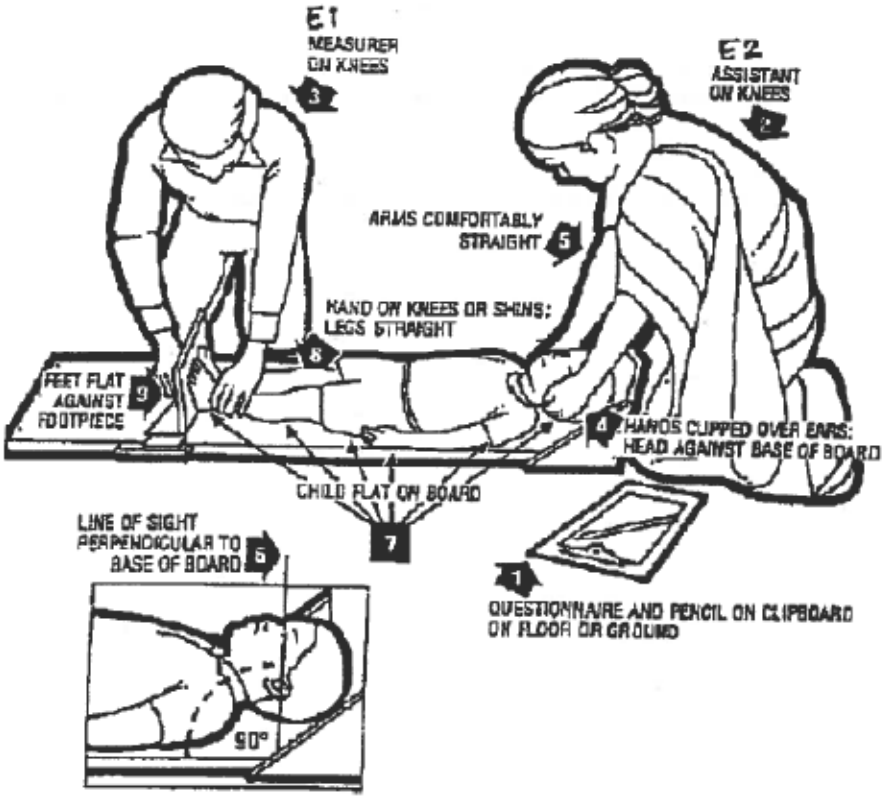
Note: when you set up the board each day, examine each of the pieces to check for damage.

Measure Length

Cover the length board with a chuck for hygiene and for the baby's comfort. Explain to the mother that she will need to place the baby on the length board and then help to hold the baby's head in place while the measurement is taken. Show her where to stand when placing the baby down (i.e. opposite you, on the side of the length board away from the tape). Also show her where to place the baby's head (against the fixed headboard) so that she can move quickly and surely without distressing the baby. When the mother understands your instructions and is ready to assist: Ask her to lay the child on his back with his head against the fixed headboard, compressing the hair.
Quickly position the head so that an imaginary vertical line from the ear canal to the lower border of the eye socket is perpendicular to the board. (The child's eyes should be looking straight up.) Ask the mother to move behind the headboard and hold the head in this position. Speed is important. Standing on the side of the length board where you can see the measuring tape and move the footboard: Check that the child lies straight along the board and does not change position. Shoulders should touch the board, and the spine should not be arched. Ask the mother to inform you if the child arches the back or moves out of position. Hold down the child's legs with the one hand and move the footboard with the other. Apply gentle pressure to the knees to straighten the legs as far as they can go without causing injury or distress.

Note: it is not possible to straighten the knees of newborns to the same degree as older children. Their knees are fragile and could be easily injured, so apply only minimum pressure.

If a child is extremely agitated and both legs cannot be held in position, measure with one leg in position. While holding the knees, pull the footboard against the child's feet. Upon reading the measurement, the examiner will clearly call out the number to the recorder. Record the child's length in centimeters to the last completed 0.1 cm. (1.0 mm). Keeping the child in place, release the sliding footboard, and prepare to repeat the measurement. Re-position the child for a second and third measurement.

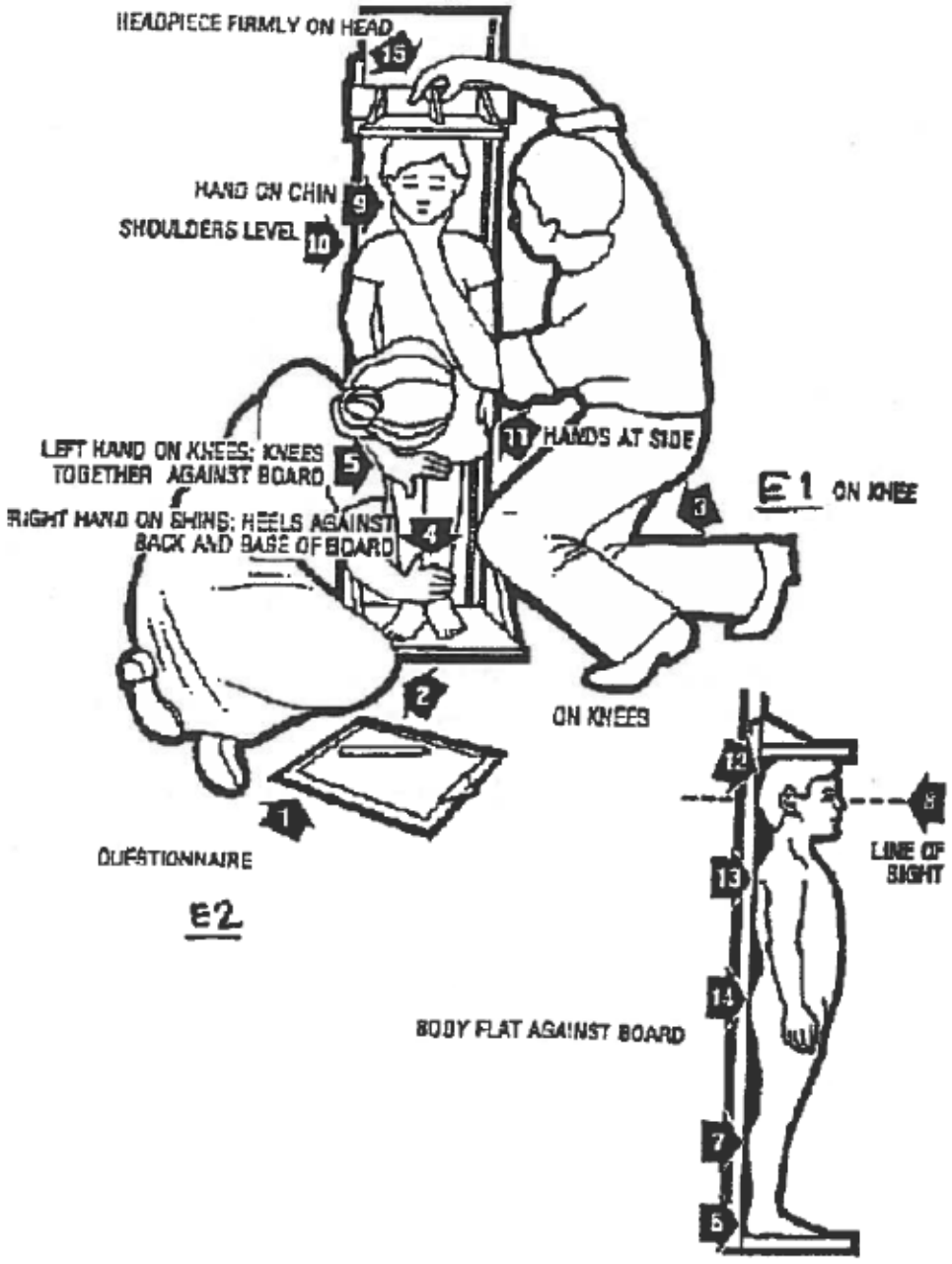


Measure Standing Height

Ensure that the height board is on level ground. Working with the mother, and kneeling in order to be at the level of the child. Help the child stand on the baseboard with the weight of the child evenly distributed on both feet. The heels of the feet are placed together with both heels touching the base of the vertical board. Place the feet pointed slightly outward at a 60 degree angle. The back of the head, shoulder blades, buttocks, calves, and heels should all touch the vertical board. Arms should hang freely by the sides of the body with the palms facing the thighs.

Note: Standing with all body parts touching the board may be difficult for some children, in which case, help the child to stand on the board with one or more contact points touching the board.

Ask the mother to hold the child's knees and ankles to help keep the legs straight and feet flat, with heels and calves touching the vertical board. Ask her to focus the child's attention, soothe the child as needed, and inform you if the child moves out of position. Position the child's head so that a horizontal line from the ear canal to the lower border of the eye socket runs parallel to the baseboard. Ask the child to inhale deeply and to stand fully erect without altering the position of the heels. If necessary, push gently on the belly to help the child stand to full height. Still keeping the head in position, use your other hand to pull down the headboard to rest firmly on top of the head and compress the hair. Upon reading the measurement, the examiner will clearly call out the number to the recorder. Record the child's height in centimeters to the last completed 0.1 cm (1.0 mm). Keeping the child in place, release the sliding headboard, and prepare to repeat the measurement. Re-position the child for a second and third measurement.


HEADPIECE FIRMLY ON HEAD
15
HAND ON CHIN
9
SHOULDERS LEVEL
10
LEFT HAND ON KNEES; KNEES TOGETHER AGAINST BOARD
5
RIGHT HAND ON SHINS; HEELS AGAINST BACK AND BASE OF BOARD
4
11 HANDS AT SIDE
E1
ON KNEE
3
2
ON KNEES
1
QUESTIONNAIRE
E2
12
8
LINE OF SIGHT
13
14
BODY FLAT AGAINST BOARD
7
6

Dismantling the ShorrBoard

1) Stand the board upright: face the board and step on the base with one foot to keep it stable.
2) Slide the head/footpiece into the base of the main board.
3) Release the clasp on the back of the extension piece and remove it. Push the clasp FLAT against the extension piece.
4) To attach the extension piece to the main board, turn the front of the extension piece inward and place it against the front of the main board. Make sure that all sides of the extension piece are straight and in line with the main board.
5) Push on the bolt that is on the back of the extension piece and screw it into the main board.
6) Put the board back inside of the carrying case for storage until your next use.

Measuring Weight

The SECA 874 scale will be used to weigh infants and children to the nearest 0.01 kg. Infants and young children can also be weighed simultaneously with their parent or guardian by the unique "mother-baby" function (parent or guardian is weighed and then the infant or child is weighed while held by the parent). Explain to the mother that we want to weigh her child to see how he or she is growing. If she has a baby or a child who is unable to stand, she will hold the child on the scale. If the child is 2 years or older/can stand alone, the child will be weighed alone. Children should be wearing only light clothing, no shoes or sandals, no hair ornaments, and no jewelry. Explain that the child needs to remove outer clothing and shoes/sandals in order to obtain an accurate weight. If the baby is wearing a diaper, the diaper should be removed. If any heavy clothes remain on the child, make a note in the Notes section.

Seca 874 Scale

1) Remove scale from bag.
2) Be sure that the scale is placed on a flat, hard, even surface. All 4 legs of the scale should make contact with the ground surface, without wobbling. It may be helpful to place a piece of plywood on the ground underneath the scale.
3) When **batt** appears in the display, you should change the batteries. Remove the old batteries and insert 6 new batteries.

Turn the power on the scale when you are ready to begin weighing. If the child is unable to stand on the scale, you will use the 2 in 1 weighing function (called tared weighing). The 2 in 1 function enables the weight of babies and small children to be determined while an adult holds them. Identify a suitable area that is flat for horizontal placement of the scale. Press the start key with no load on the scale. The scale is ready for use when it sets to 0.00. If necessary, switch the weight display to KG: hold down the 2 in 1 key for about 3 seconds. Press the start key with no load on the scale. Wait until the display shows 0.00.

Ask the adult to remove his/her shoes and stand in the middle of the scale without the child. S/he should remove any long garments, as these can cover the display and also lead to variable measurements. After the adult's weight appears on the display, tell him/her to remain standing on the scale. Press the 2 in 1 key to activate the function. The scale stores the weight of the adult and the display returns to zero. When 0.00 and NET appear in the display, hand the child to the adult. The scale will determine the weight of the child. Once the value is stable for about 3 seconds, the weight is measured.

Note: If an adult is very heavy (e.g. more than 100 kg) and the baby's weight is relatively low (e.g. less than 2.5 kg), the baby's weight may not register on the scale. In such cases, have a lighter person hold the baby on the scale.

The positioner will clearly call out the child's weight to the recorder. Record the child's weight to the nearest 0.01 kg. Repeat the measurement 2 more times. Note that only the baby needs to removed from the scale; the adult should remain on the scale the entire time. To turn off the 2 in 1 function, press the 2 in 1 key. The 2 in 1 function remains on until you press the 2 in 1 key again, or until the scale switches off automatically. If several children are to be weighed consecutively with the same adult holding the babies, it is important that this person's weight does not change (e.g. due to a piece of clothing being removed/added). If the child is able to stand on the scale, you will weigh the child alone. Talk with the child about the need to stand still. Communicate with the child in a sensitive, non-frightening way. Press the start key with no load on the scale. The scale is ready for use when it sets to 0.00. Ask the child to stand in the middle of the scale. Once on the scale, the child must stand still. The HOLD function is automatically activated for weights over 1.5 kg/3.3 lbs. The display flashes until a stable weight has been measured. The display is then frozen until the next weighing operation.

Note: If the child jumps on the scale or won't stand still, you will need to use the tared weighing procedure instead.

The positioner will clearly call out the child's weight to the recorder. Record the child's weight to the nearest 0.01 kg. Repeat the measurement 2 more times. Have the child move completely off the scale and then stand again on the scale. If no further weighing operations are performed, the scale switches off automatically after 2-3 minutes.

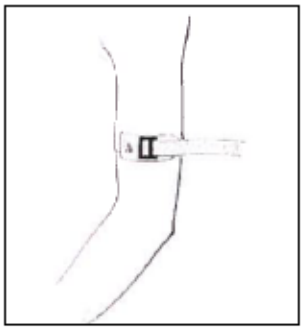
Scale Calibration

In order to monitor the calibration of the scales over time, each team will weigh a 5 kg test weight at the beginning and end of the day. This measurement will take place at the site of the scale storage so that the weights do not need to be carried to the field. In some cases, these weights will be kept in a project vehicle.

Measuring Mid-Upper Arm Circumference (MUAC)

The child's MUAC will be measured one time. MUAC measurements will be taken at the midpoint of the left arm between the tip of the shoulder and the tip of the elbow using non-stretch MUAC tapes.

First, find the approximate midpoint of the upper arm. Have the child stand up straight with feet together, and the right arm bent 90 degrees at the elbow, palm facing up. The examiner is positioned behind the child. If it is helpful, mark the midpoint with a permanent marker. To measure mid-upper arm circumference, have the child stand up straight with the arms relaxed at the sides. The examiner will stand facing the child's right side. The measuring tape is placed around the upper arm at the marked point. Wrap the tape around the arm, pulling it to lie flat against the surface of the skin. Be careful not to pull the tape too tightly (to compress the skin).



The line for reading measurements is clearly labeled on the MUAC tape (READ (cm)). Read the number aligned with the measurement line on the tape Upon measuring, the examiner will clearly call out the number to the recorder. Record to the nearest 0.1 cm (0.1 cm = 1 mm). Keeping the child in place, release the MUAC strip.

If the MUAC is <11.5cm refer the child to the nearest health center.

6. COGNITIVE DEVELOPMENT ASSESSMENT

The Developmental Milestones Checklist (DMC-III) will be used to evaluate the development of motor and language skills in 240 children at baseline in this study. The DMC-III includes three subscales: gross motor, fine motor, and language. The original version of the DMC-III was designed for children aged 3 to 24 months. The updated DMC-III includes additional items to assess children aged 2 to 8 years. The DMC-III requires parent interview for all items and includes optional direct observation of the child for all items. For this study, data collectors will include both parent interview and direct observation for all items where possible.

Materials for observation items:

- Pens
- White paper
- A ball (child-size football/soccer ball)
- A small toy
- Small grains in a box
- A pull-toy
- A color sheet printed with boxes of the following colors : blue, green, yellow, brown, purple, white, grey, orange, red, black, and pink

- 5 coins

Preparing the assessment session for observation items:

2.1 Before the assessment session

a) **Check** that you have all of the **equipment** and record **forms** required using the checklist.

2.2 When you arrive at the child's house

a) **Greet** the mother or caregiver accompanying the child.
b) **Introduce** yourself and outline the purpose of the visit
c) **Approach** the child for the first time in the presence of the mother / caregiver
d) **Observe** carefully the child's reaction to yourself and the situation as it unfolds.
e) **Confirm** that the child is in good **health**. **DO NOT ASSESS A CHILD WHO IS UNWELL**. If the child is sick, conduct the interview portion of the visit and make an appointment to return at a later date to conduct the observation items.

2.3 Guidelines for the testing location

a) The testing location should be a place where there is shade, flat ground, maximum privacy, and space for movement.
b) Take time to select the largest, quietest location that allows you to run around. You can sit in one place and run around elsewhere.
c) **Remove** possible **distracters** before starting to assess.
d) If possible, position the child **facing a wall** to minimize distractions.
e) Make sure the child and mother are settled and **comfortable**.
f) Take good care to ensure the safety of the children. Look out for potential hazards.
g) If others gather to watch, ask them politely to leave. Explain that the child might be shy if he or she is being watched by others. If necessary, ask for the mother's help to disperse the crowd.

2.4 Setting up the equipment.

a) **Arrange** equipment where it will be easily accessible to you.
b) **Explain** to the family member who accompanies the child their role. To
   - provide comfort to their child by their presence
   - encourage the child to participate
   - (if necessary) to help communicate the instructions to the child Help them to understand the game, but NOT to assist the child to complete tasks.

2.5 Settling the child

a) **Use** the name the child is called by throughout the session. Whenever you need

to get the
child's attention to ask a question or give an instruction, say his or her name.

b) **Re -introduce** yourself to the child
c) **Invite** the child to participate in an activity.
d) **Engage** the child with some of the equipment/activities. This may include a warm up
activities, separate from the main assessment.
e) In general, children respond well when we smile, are enthusiastic, playful, and affectionate.

2.6 General instructions for administering fine and gross motor items:

a) **First**, demonstrate the task.
b) **Second**, help the child to do the task. Give support where necessary until you are certain that the child can complete the task unsupported. Remove any support provided in stages.
c) **Third**, tell the child to do the task alone, without assistance.

2.7 During assessment.

a. Carry out all activities on flat and even ground.
b. Child should be in a clear space away from furniture / walls.
c. The inclusion of mother and siblings in these activities often aids and encourages the target child to feel less inhibited.
d. **Give** precise and clear instructions to the child, following manual guidelines.
e. **Move** from one task to the other in a systematic way.
f. **Pay attention** to the child at all times.
g. **Praise** the child for effort, NOT success in an item.
h. **Return** assessment material to the box on completion of the item. Take out the material for one item at a time and replace it when you have finished. Sometimes you may take out the material for the next item before replacing the material for the previous item, if this is necessary to keep the child's attention. However, in this case, replace the material for the previous item immediately after engaging the child's attention with the new item.
i. **Take a break** if the child gets tired or becomes distressed. In general, it is a good idea to take a break at least every 30 minutes.
j. **If necessary, remind** the accompanying family member of their role in the assessment.

Do NOT:

1) **Crowd** the assessment area with used items, this will distract the child.
2) **Hurry** the child.

2.8 Strategies for handling children who refuse

a) Have the mother demonstrate the task and encourage the child to do it

b) Involve other children who are around, perhaps an older sibling. Have the other child do it and see if the child will do the task along with another child.
c) Give the child a drink and a break, let the child rest or play for a few minutes
d) Promise to give the child a sweet if he/she does the task

2.9 Ending the assessment

a) **Formally** end the session by thanking everyone.
b) **Before you leave the house**
    i. **Read** through your form carefully.
    ii. **Confirm e**ach section/ checklist is filled fully.
c) Check all equipment is packed properly. (Use checklist to help you.)

2.10 Before the next session

CLEAN AND DISINFECT THE EQUIPMENT EVERY DAY.

Administration procedures

In each subscale, the items are arranged in order from the earlier acquired skill (for example, sits supported) to the later acquired skill (for example, sits alone on the floor). The arrows in the left-had margin show the start points for the child's age. Start at the item appropriate for the child's age. If the child scores 0, go to the previous start point. If the child scores 1 or 2, continue to the next item. Stop after 4 consecutive scores of 0. Refer to the DMC-III Manual for a detailed description of how to administer each item, including probing questions to use if the interviewee has difficulty understanding the question. The DMC-III flipchart includes pictures, which should be used in conjunction with the questions for the two motor subscales.

7. MICROBIOLOGICAL METHODS

Participant names will not be included in laboratory records and samples will be de identified. Therefore, laboratory personnel will be masked, preventing identification of the individuals infected. Lab results will not be available for weeks, if not months. Thus, all individuals will be treated according to their study arm, whether or not their lab tests reveal evidence of infection.

Preparation of Zymo DNA/RNA Shield™

Manufacturer's Product Description

DNA/RNA Shield™ ensures nucleic acid stability during sample storage/transport at ambient temperatures, preserving the genetic integrity and expression profiles of samples. There is no need for refrigeration or specialized equipment. DNA/RNA Shield™ effectively lyses cells and inactivates nucleases and infectious agents (virus), and it is compatible with various collection and storage devices (vacuum tubes, swabs, nasal, buccal, fecal, etc.).

Materials Required

Product	Supplier	Catalog No.
DNA/RNA Shield™ 1X solution	Zymo Research Corp.	R1100-50 (50 ml) R1100-250 (250 ml)
2ml Loop Screw Cap Micro Tube, PCR Performance Tested (DNA-, DNase-, RNase-, & PCR inhibitor-free)	Sarstedt Inc.	72.694.416
Insert for Loop Screw Cap, Violet	Sarstedt Inc.	65.713.007

Procedure

1. Prepare tubes by adding violet cap inserts to the top of each tube (these are for color coding purposes to help distinguish the DNA/RNA Shield solution from other media being used in the field).
2. Before aliquoting the DNA/RNA Shield, gently mix the solution by either swirling it or inverting the bottle a few times (trying not to create too many bubbles) to ensure that the solution is homogenous. In a clean biosafety cabinet, use sterile technique to aliquot 1 ml of the solution into each 2 ml tube.
3. When you have finished aliquoting, make sure that the cap on each tube has been tightened to prevent tubes from leaking during transport.

Storage and Shelf Life

Store prepared tubes at room temperature (20°C-25°C) until use; do not freeze solution prior to collecting samples. Zymo guarantees the shelf-life of DNA/RNA Shield ™ up to 1 year from the date of receipt. Therefore, if the solution is aliquoted right away, then prepared tubes should be good for 1 year. The shelf-life may be longer, depending on storage conditions.

Methods for Resistance Testing

Isolation & Identification of Organisms Using Microbiological Techniques

S. pneumoniae will be isolated from nasopharyngeal swabs using blood and strep select plate, chocolate, and chocolate with bacitracin media. We will identify organisms using gram stain. Standard microbiological handbooks should be referenced for more detailed information.

Laboratory Results Reporting

All lab results will be kept in computer files as well as in hard-copy form by the Microbiologist.

Description of Identification Tests:

We will follow the standard quality control methods already in place at the laboratory. For example, lab staff will perform positive and negative growth controls for all media, and positive controls for all stains and identification tests at a pre-determined schedule.

Catalase Test:
A few drops of 3% hydrogen peroxide are added to colonies removed from agar and smeared onto a clean glass slide, and immediately observed for the release of oxygen or "bubbling." Bubbling is interpreted as the presence of catalase. Lack of bubbling is a negative test.

Tube Coagulase:
Coagulase plasma is inoculated with an isolated staphylococcal colony and incubated for up to 24 hours at 37°C. Any degree of clot formation is a positive reaction and no clot is a negative reaction.

Latex Agglutination:
A few staphylococcal colonies are mixed with the latex agglutination reagent and observed for agglutination. A positive reaction is agglutination of the latex particles, while no agglutination is a negative reaction.

Optochin Test:
An optochin disc is placed onto a blood agar subcultured plate that has been inoculated with a pure culture of the test organism. Plates are incubated for 24 hours at 35°C in CO_2. If a zone of inhibition of 14 mm or more is formed, then it's *S. pneumoniae*.

Desoxycholate (10%) Test:
A drop of reagent is added to a well-isolated colony on a blood agar plate. Colonies of *S. pneumoniae* will disintegrate after 30 minutes.

Satelliting Growth on Blood Agar:
Colonies resembling Haemophilus are subcultured to a non-selective blood agar plate and cross streaked with Coagulase negative Staphylococcus. Plates are incubated in CO_2 overnight and examined for satellite growth around Staphylococcus.

Microbiological Resistance Testing for Swabs
Resistance testing will be performed using the Kirby Bauer disc diffusion assay or E-test strips (to determine MIC's).

Quality Control
E. coli ATCC 25922, *S. pneumoniae* ATCC 49619, *H. influenzae* ATCC 49247, *S. aureus* ATCC 25923 and 29213 are used for quality control testing of antibiotic discs and E-test strips.

Kirby Bauer Assay
A suspension of the isolate is prepared and turbidity adjusted until it reaches the 0.5 McFarland standard. The suspension is used to inoculate the appropriate susceptibility agar plate for

confluent growth. The plate is allowed to dry. Antimicrobial disks are placed on the agar plates with sterile forceps or tweezers; disks may not be moved once they have touched the plates. After the disks are placed on the plate, the plates are inverted and incubated at 35°C for 16 to 18 hours. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 2) are measured and recorded in millimeters. The measurements can be made with a ruler or sliding caliper on the undersurface of the plate without opening the lid. The zones of growth inhibition will be compared with the zone-size interpretative table and recorded as susceptible, intermediate, or resistant to each drug tested according to CLSI guidelines (see Appendix).

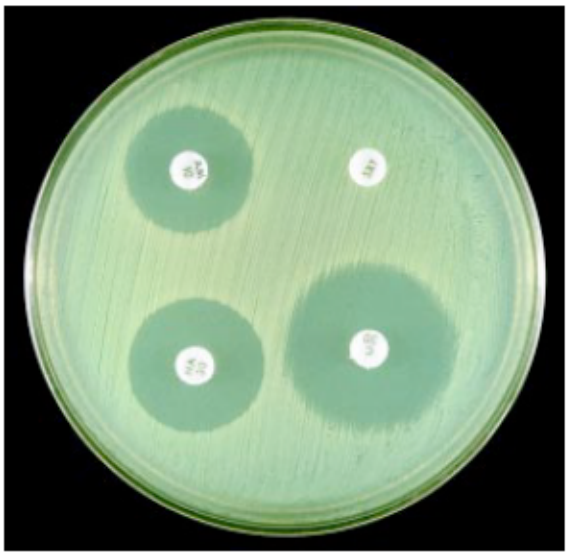


Figure 2. Results of the disk diffusion assay. This ***Shigella*** isolate is resistant to trimethoprim-sulfamethoxazole and is growing up to the disk (SXT), the zone of which is recorded as 6 mm.

E-test strips.

In addition, azithromycin Minimum Inhibitory Concentrations (MIC) are determined using E-test strips. A suspension of the isolate is prepared and turbidity adjusted until it reaches the 0.5 McFarland standard. The suspension is used to inoculate the appropriate susceptibility agar plate for confluent growth. The plate is allowed to dry. E-test strips are placed on the agar plates with sterile forceps or tweezers; strips may not be moved once they have touched the plates. After the strips are placed on the plate, the plates are inverted and incubated at 35°C for 20 to 24 hours. The MIC is read where the ellipse intersects the MIC scale on the strip. The MIC will be compared with the interpretative table according to CLSI guidelines (see Appendix).

8. PROTECTION OF HUMAN SUBJECTS

Before the study begins, each site will obtain formal ethical approval from their respective national ethics committee. In addition, local staff will approach community leaders to describe the study and answer any questions. Study staff will proceed only if local leadership consents to participate. For Study One, we will obtain verbal consent from a parent or guardian of all study participants. For Study Two, we will obtain written consent from a parent or guardian for all

study activities, including treatment and examinations. Children will only be included in the study following receipt of consent from the parent or guardian. If, at any time, a parent or guardian elects to withdraw a family member from the study, they will be free to do so. Children with wasting or stunting will be referred for appropriate treatment by trained study personnel to the nearest health center.

Risks and Benefits of Study Procedures

Swabbing Procedures

There are minimal risks to the participant who receives nasopharyngeal, and nares swabbing. Participants may experience some temporary discomfort, but the swabbing involves minimal risk. Any adverse effects, such as nose-bleeds, will be treated immediately by the examiners. Other health care will be provided at no cost to the study participant if necessary to address a study-related adverse health event.

Rectal swabs and stool samples: Rectal swabs are a simple procedure with very minimal risk. Stool sample collection in a non-invasive procedure with no associated risks.

Anthropometric Measurements

There are minimal risks associated with the measuring board, scale, or MUAC tapes aside from anxiety during the measurements. Examiners will do their best to ensure that the parent/guardian of the child understands the process of assessing anthropometric measurements. The examiners will attempt to minimize discomfort for all study participants before, during, and after the measurements are taken. Children with wasting or stunting will be referred for appropriate treatment at the nearest health center.

9. APPENDICES

10. REFERENCES

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